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(54) A METHOD FOR THE PRODUCTION OF D-RIBOSE

(71) We, TAKEDA YAKUHIN KOGYU KABUSHIKI KAISHA, also known as TAKEDA CHEMICAL INDUSTRIES LTD. of 27, Doshomachi 2-chome, Higashi-ku, Osaka, Japan, a body corporate organised under the laws of Japan, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:

This invention relates to a method for the production of D-ribose.

This invention provides a method for the production of D-ribose, which comprises inoculating a D-ribose-producing microorganism of the genus *Bacillus* which requires L-tyrosine, L-tryptophan and L-phenylalanine for its growth and which cannot utilize D-gluconic acid, L-arabinose and D-ribose, into a culture medium containing L-tyrosine, L-tryptophan and L-phenylalanine, each in an amount of more than 100 γ /ml., in addition to assimilable carbon and nitrogen sources; incubating the culture medium until D-ribose is accumulated therein; and recovering the D-ribose thus accumulated from the culture medium.

D-ribose is important, for example, as a starting material in the synthesis of riboflavin compounds and as a raw material in the production of nucleotide condiments which have recently been developed.

Heretofore, several methods have been known for producing D-ribose employing microorganisms, but they are hardly feasible commercially, because they give only small amounts of D-ribose.

According to the present invention, the incubation of certain microorganisms of the genus *Bacillus* brings about the accumulation of D-ribose in a remarkably large amount in the culture medium, and D-ribose accumulated in this way is easily recoverable from the culture medium.

The invention therefore provides a

method for the production of D-ribose which can be put into practice on an industrial scale with a good yield.

A D-ribose-producing microorganism of the genus *Bacillus* which requires L-tyrosine, L-tryptophan and L-phenylalanine for its growth is inoculated into a culture medium containing L-tyrosine, L-tryptophan and L-phenylalanine, and the culture medium is incubated (hereinafter, the microorganisms mentioned above are simply referred to as "the Microorganisms.").

The Microorganisms are derived from wild type strains by means of conventional techniques for mutations or they are isolated from wild type strains.

The mutation may be effected by subjecting wild type strains belonging to the genus *Bacillus* to treatment with, for example, ultraviolet rays, X-rays, gamma rays, or mutagens such as nitrosoguanidine.

The Microorganisms are, for example, the mutants of *Bacillus pumilus* such as *Bacillus pumilus* No. 503 (IFO 12600), *Bacillus pumilus* No. 537 (IFO 12601) and *Bacillus pumilus* No. 558 (IFO 12602), and the mutants of *Bacillus subtilis* such as *Bacillus subtilis* No. 429 (IFO 12603) and *Bacillus subtilis* No. 483 (IFO 12604).

Representative microbiological characteristics of these microorganisms are that they cannot utilise for their growth D-gluconic acid, L-arabinose or D-ribose as the carbon source and that they require for their growth L-tyrosine, L-tryptophan and L-phenylalanine. In other microbiological aspects, their properties are identical with those set forth in Bergey's manual of Determinative Bacteriology, 620-622 (1957).

For the purpose of the industrial production of D-ribose, the cultivation of the Microorganisms is preferably carried out in a liquid culture medium. The incubation is carried out generally as a submerged pro-

cess under aeration and agitation, employing a culture medium containing L-tyrosine, L-tryptophan and L-phenylalanine.

Although L-tyrosine, L-tryptophan and L-phenylalanine may be added to the culture medium in the respectively isolated form, natural substances containing them such as dried yeast, peptone, meat extract, casamino-acid and corn steep liquor, and mixtures thereof may also be employed.

L-Tyrosine, L-tryptophan and L-phenylalanine should be added to the culture medium in sufficient amounts for the growth of the Microorganisms. L-tyrosine, L-tryptophan and L-phenylalanine are added to the culture medium so as to make their respective concentrations higher than 100 γ /ml., but preferably lower than 5000 γ /ml.

As the assimilable carbon source, use may be made of one or more compounds such as monosaccharides, e.g. D-glucose, D-fructose, D-mannose, sorbitol and D-mannitol, disaccharides, e.g. sucrose and maltose, various polysaccharides, e.g. dextrin, soluble starch, blackstrap molasses and the like.

As the assimilable nitrogen source, for example, use may be made of inorganic nitrogen compounds such as ammonium sulphate and ammonium nitrate, as well as organic nitrogenous compounds such as urea. Furthermore, a small quantity of inorganic salts such as magnesium sulphate, calcium phosphate and calcium carbonate may be used.

The incubation temperature should be controlled so as to accumulate the desired substance in the maximum amount. Generally, the temperature is adjusted within a range such as from 25°C to 45°C.

Incubation is continued until the maximum amount of D-ribose is accumulated in the culture medium. Although the period required for the maximum accumulation of D-ribose depends upon various factors, it usually falls between 24 to 120 hours from the start of the incubation.

The culture broth at the completion of cultivation contains a large amount of D-ribose. Since a major portion of the D-ribose thus produced is accumulated in the liquid phase of the broth, it is recommended to begin with to filter off the cells and then to recover the D-ribose from the filtrate. For example, the D-ribose can be recovered by desalting the filtrate with an ion exchange resin and decolorising it with the aid of a carbon powder column.

When the culture broth at the end of cultivation contains raw material carbohydrates in addition to the desired D-ribose, those raw material carbohydrates may be removed through various conventional processes, e.g. by treating the broth with glucose-oxidase in the case of glucose being

present or with baker's yeast which does not utilise D-ribose but does utilise other carbohydrates alone.

The following examples are merely intended to illustrate presently preferred embodiments of this invention and not to restrict the scope thereof.

In the present specification the abbreviation IFO "Institute for Fermentation Osaka". The percentages are weight/volume unless otherwise described

Example 1

Bacillus pumilus mutant No. 503 (IFO 12600), which was derived from *Bacillus pumilus* (ATCC No. 7061) by irradiation with ultraviolet rays followed by subjection to penicillin screening (Experientia, 66, 41 (1960)) and to the replica plating method (Journal of Bacteriology, 63, 399 (1952)), is inoculated into 500 ml. of a medium containing 2% of soluble starch, 2% of corn steep liquor, 0.3% of dipotassium hydrogen phosphate and 0.1% of potassium dihydrogenphosphate. The medium is incubated at 28°C for 24 hours to prepare a seed culture. This seed culture is inoculated into 30 liters of a medium (hereinafter called Medium A) which contains 12.5% of glucose, 2.5% of dried yeast, 1.5% of ammonium sulphate, 0.5% of calcium secondary phosphate, 0.5% of calcium tertiary phosphate and 1.0% of calcium carbonate. The medium is incubated at 32°C for 66 hours under constant aeration and agitation. The above procedure yields 28.5 mg./ml. of D-ribose in the culture broth.

The above D-ribose-containing broth is passed through a column of activated carbon (15 cm by 100 cm) and the effluent is concentrated to about 5 liters under reduced pressure and is incubated after the addition of 10% of baker's yeast at 37°C for 1 hour. After the yeast cells have been removed by filtration, the concentrate is further passed, for desalting, through layers of respectively cation-exchange resins (commercially available as Amberlite (trade mark) IR-120) and anion-exchange resins (commercially available as Amberlite IR-A-400), and the effluent is concentrated to 1 liter. To this concentrate, 1 liter of ethyl alcohol is added, whereby 620g. of crude crystals of D-ribose are obtained.

Example 2

Bacillus pumilus mutant No. 537 (IFO 12601) prepared in the same manner as in Example 1 is inoculated into 500 ml. of a medium containing 2% of sorbitol, 2% or corn steep liquor, 0.3% of dipotassium hydrogenphosphate and 0.1% of potassium dihydrogenphosphate. The medium is incubated at 32°C for 24 hours to prepare a seed culture. The seed culture is then inoculated into 30 liters of a medium consisting of Medium A and 150 γ /ml. of L-phenyl-

alanine. This medium is then incubated at 37°C for 55 hours with aeration and agitation. The above procedure yields 30.5 mg./ml. of D-ribose in the culture broth.

- 5 This D-ribose-containing broth is treated in the same manner as in Example 1 to yield 693g. of crude crystals of D-ribose

Example 3

- 10 *Bacillus pumilus* mutant No. 558 (IFO 12602) prepared in the same manner as in Example 1 is inoculated into 500 ml. of a medium of the same composition as was used for the preparation of the seed culture in Example 2. The medium is incubated at 37°C for 24 hours under shaking to prepare a seed culture.

- 15 This seed culture is inoculated into 30 liters of Medium A to which 50 γ/ml. of L-tyrosine are added. The medium is incubated at 37°C for 80 hours under aeration and agitation. The above procedure yields 29.3 mg./ml. of D-ribose in the culture broth. This culture broth is treated in the same manner as in Example 1, whereby 25 620g. of crude crystals of D-ribose are obtained.

Example 4

- 30 *Bacillus subtilis* mutant No. 429 (IFO 12603), which is prepared by irradiation of *Bacillus subtilis* (ATCC No. 6051) with ultraviolet rays followed by subjection to penicillin screening (described above) and the replicas plating method (described above), is inoculated into 500 ml. of a medium containing 2% of soluble starch, 2% of corn steep liquor, 0.3% of dipotassium hydrogenphosphate and 0.1% of potassiumdihydrogen phosphate. The medium is incubated at 32°C for 24 hours. This 40 seed culture is then inoculated into 30 liters of a medium containing 10% of soluble starch, 1.5% of dried yeast, 1.5% of ammonium sulphate, 0.5% of calcium tertiary phosphate, 0.5% of calcium secondary phosphate and 1.0% of calcium carbonate. This medium is incubated at 32°C for 72 hours under aeration and agitation. The above procedure yield 26.5 mg./ml. of D-ribose in the culture broth.

- 50 This culture broth is filtered with a filter aid to remove the cells, and the filtrate is passed through a column (20cm by 100cm) of acid-pretreating carbon powder. The effluent is concentrated to about 4.5 liters under reduced pressure which is incubated after the addition of 10% of baker's yeast at 37°C for 1 hour. After the yeast cells are removed by filtration, the concentrate is passed, for desalting, through layers of 60 cation exchange resins (commercially available as Amebrlite IR-120) and anion exchange resins (commercially available as Amberlite IR-A-400), and the effluent is further concentrated to 1 liter under reduced 65 pressure. 1.5 liters of ethyl alcohol

are added, to the concentrate whereby 558g. of crude crystals of D-ribose are obtained.

Example 5

Bacillus subtilis is irradiated with ultraviolet rays to yield *Bacillus subtilis* mutant No. 483 (IFO 12604). This mutant is inoculated into 500 ml. of Medium A at 30°C for 20 hours. The resulting seed culture is inoculated into 30 liters of a medium containing 10% of D-glucose, 2.0% of dried yeast, 1.8% of ammonium sulphate, 0.5% of calcium phosphate, 1.0% of calcium carbonate and 100γ/ml. of L-phenylalanine. The medium is incubated at 32°C for 68 hours. The above procedure yields 29.5 mg./ml. of D-ribose in the culture broth. The broth is treated in the same manner as in Example 1 to yield 615 g. of crude crystals of D-ribose.

WHAT WE CLAIM IS:

1. A method for the production of D-ribose, which comprises inoculating a D-ribose-producing microorganism of the genus *Bacillus* which requires L-tyrosine, L-tryptophan and L-phenylalanine for its growth and which cannot utilize D-gluconic acid, L-arabinose and D-ribose, into a culture medium containing L-tyrosine, L-tryptophan and L-phenylalanine, each in an amount of more than 100γ/ml., in addition to assimilable carbon and nitrogen sources; incubating the culture medium until D-ribose is accumulated therein; and recovering the D-ribose from the culture medium.
2. A method according to claim 1, wherein the amount is less than 5000γ/ml.
3. A method according to claim 1, wherein the microorganism is incubated in the culture medium at a temperature of from 25°C to 45°C under aerobic conditions.
4. A method according to claim 1, wherein the microorganism is a mutant of *Bacillus pumillus*.
5. A method according to claim 4, wherein the microorganism is *Bacillus pumilus* No. 503 (IFO 12600).
6. A method according to claim 4, wherein the microorganism is *Bacillus pumilus* No. 537 (IFO 12601).
7. A method according to claim 4, wherein the microorganism is *Bacillus pumilus* No. 558 (IFO 12602).
8. A method according to claim 1, wherein the microorganism is a mutant of *Bacillus subtilis*.
9. A method according to claim 8, wherein the microorganism is *Bacillus subtilis* No. 429 (IFO 12603).
10. A method according to claim 8, wherein the microorganism is *Bacillus subtilis* No. 483 (IFO 12604).
11. A method according to claim 1 substantially as herein described with ref-



alanine. This medium is then incubated at 37°C for 55 hours with aeration and agitation. The above procedure yields 30.5 mg./ml. of D-ribose in the culture broth.

5 This D-ribose-containing broth is treated in the same manner as in Example 1 to yield 693g. of crude crystals of D-ribose

Example 3

10 *Bacillus pumilus* mutant No. 558 (IFO 12602) prepared in the same manner as in Example 1 is inoculated into 500 ml. of a medium of the same composition as was used for the preparation of the seed culture in Example 2. The medium is incubated at 37°C for 24 hours under shaking to prepare a seed culture.

15 This seed culture is inoculated into 30 liters of Medium A to which 50 γ/ml. of L-tyrosine are added. The medium is incubated at 37°C for 80 hours under aeration and agitation. The above procedure yields 29.3 mg./ml. of D-ribose in the culture broth. This culture broth is treated in the same manner as in Example 1, whereby 25 620g. of crude crystals of D-ribose are obtained.

Example 4

30 *Bacillus subtilis* mutant No. 429 (IFO 12603), which is prepared by irradiation of *Bacillus subtilis* (ATCC No. 6051) with ultraviolet rays followed by subjection to penicillin screening (described above) and the replicas plating method (described above), is inoculated into 500 ml. of a medium containing 2% of soluble starch, 2% of corn steep liquor, 0.3% of dipotassium hydrogenphosphate and 0.1% of potassiumdihydrogen phosphate. The medium is incubated at 32°C for 24 hours. This 40 seed culture is then inoculated into 30 liters of a medium containing 10% of soluble starch, 1.5% of dried yeast, 1.5% of ammonium sulphate, 0.5% of calcium tertiary phosphate, 0.5% of calcium secondary phosphate and 1.0% of calcium carbonate. This medium is incubated at 32°C for 72 hours under aeration and agitation. The above procedure yield 26.5 mg/ml. of D-ribose in the culture broth.

50 This culture broth is filtered with a filter aid to remove the cells, and the filtrate is passed through a column (20cm by 100cm) of acid-pretreating carbon powder. The effluent is concentrated to about 4.5 liters under reduced pressure which is incubated after the addition of 10% of baker's yeast at 37°C for 1 hour. After the yeast cells are removed by filtration, the concentrate is passed, for desalting, through layers of 60 cation exchange resins (commercially available as Amebrlite IR-120) and anion exchange resins (commercially available as Amberlite IR-A-400), and the effluent is further concentrated to 1 liter under reduced 65 pressure. 1.5 liters of ethyl alcohol

are added, to the concentrate whereby 558g. of crude crystals of D-ribose are obtained.

Example 5

Bacillus subtilis is irradiated with ultraviolet rays to yield *Bacillus subtilis* mutant 70 No. 483 (IFO 12604). This mutant is inoculated into 500 ml. of Medium A at 30°C for 20 hours. The resulting seed culture is inoculated into 30 liters of a medium containing 10% of D-glucose, 2.0% of dried 75 yeast, 1.8% of ammonium sulphate, 0.5% of calcium phosphate, 1.0% of calcium carbonate and 100γ/ml. of L-phenylalanine. The medium is incubated at 32°C for 68 hours. The above procedure yields 29.5 mg./ml. 80 of D-ribose in the culture broth. The broth is treated in the same manner as in Example 1 to yield 615 g. of crude crystals of D-ribose.

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WHAT WE CLAIM IS:

1. A method for the production of D-ribose, which comprises inoculating a D-ribose-producing microorganism of the genus *Bacillus* which requires L-tyrosine, 90 L-tryptophan and L-phenylalanine for its growth and which cannot utilize D-gluconic acid, L-arabinose and D-ribose, into a culture medium containing L-tyrosine, L-tryptophan and L-phenylalanine, each in an 95 amount of more than 100γ/ml., in addition to assimilable carbon and nitrogen sources; incubating the culture medium until D-ribose is accumulated therein; and recovering the D-ribose from the culture medium. 100

2. A method according to claim 1, wherein the amount is less than 5000γ/ml.

3. A method according to claim 1, wherein the microorganism is incubated in the culture medium at a temperature of 105 from 25°C to 45°C under aerobic conditions.

4. A method according to claim 1, wherein the microorganism is a mutant of *Bacillus pumillus*. 110

5. A method according to claim 4, wherein the microorganism is *Bacillus pumilus* No. 503 (IFO 12600).

6. A method according to claim 4, wherein the microorganism is *Bacillus 115 pumilus* No. 537 (IFO 12601).

7. A method according to claim 4, wherein the microorganism is *Bacillus pumilus* No. 558 (IFO 12602).

8. A method according to claim 1, 120 wherein the microorganism is a mutant of *Bacillus subtilis*.

9. A method according to claim 8, wherein the microorganism is *Bacillus 125 subtilis* No. 429 (IFO 12603).

10. A method according to claim 8, wherein the microorganism is *Bacillus subtilis* No. 483 (IFO 12604).

11. A method according to claim 1 substantially as herein described with ref- 130



erence to any of the specific examples.

12. D-ribose when produced by a
method as claimed in any of claims 1 to 11.

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